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APPLICATION

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TITLE: BIOLOGICAL CONTROL OF NEMATODES

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BIOLOGICAL CONTROL OF NEMATODES

TECHNICAL FIELD

The present invention relates to methods and materials for controlling nematodes.

PRIOR ART

Several thousand species of nematodes, sometimes called eel worms, are known. Numerous nematodes attack and parasitize humans and animals and cause disease. Additionally, several hundred species are known to feed on living plants. Certain of these are reviewed by Agrios in "Plant Pathology - 3rd Ed" Pub Academic Press Inc, see Chapter 15 therein.

Methods of controlling nematodes and their associated diseases include cultural practices; biological methods, e.g. use of resistant varieties; physical methods, e.g. heat; and use of chemical agents.

Patent application WO 92/19739 (Mycogen) relates to genes and gene fragments from *Bacillus thuringiensis* which have nematocidal activity. These generally encode crystal toxins from particular strains.

Patent application EP 0 303 426 (Mycogen) also relates to strains of *B. thuringiensis* which have nematocidal activity.

Patent application EP 0 171 381 (Monsanto) relates to particular soil bacteria which are capable of proliferating in an environment which is infested with

nematodes such as pseudomonads which colonise the surface of plant roots. The basis for the controlling activity appears to stem from glycosidase enzymes which are hypothesised to directly inhibit the nematodes.

Notwithstanding these disclosures, there is an ongoing requirement for materials which have nematocidal activity, for instance for use in crop protection or nematode-mediated disease control.

Patent application PCT/WO 99/22598 (University of Reading) published 14 May 1999 claims a biopesticide for the control of insect pests or plant parasitic nematodes or both, which comprises as an effective agent a species of bacteria which is a symbiont of an entomopathogenic nematode.

DISCLOSURE OF THE INVENTION

The present inventors have established that species of bacteria which in nature are associated symbiotically with entomopathogenic nematodes, can in fact be utilised to control nematodes, and in preferred forms of the invention, to kill them. The bacteria themselves can be employed, or nematode control agents can be used which are derived from such bacteria. In one aspect of the invention, the present invention employs bacteria which are engineered and thus not naturally occurring, or nematode control agents which are derived from natural or non-natural bacteria.

It has been reported that certain bacterial species such as *Xenorhabdus* and *Photorhabdus* can be used to control insects, see e.g. PCT/WO 98/08388 of MAFF, PCT/WO 97/17432 of WARF, and PCT/WO 99/42589 of Novartis. An effect against nematodes had not previously been demonstrated.

The symbiotic bacteria used in the present invention are isolatable from nematodes or the insects which the nematodes attack, and differ fundamentally in terms of life-style and activity from those soil bacteria such as *B. thuringiensis* or pseudomonads which have previously been suggested as being nematocidal.

Indeed, *prima facie*, it seems highly unlikely that nematode symbiotes might possess nematocidal activity. However, in the light of the present disclosure, a number of possible explanations for the observed activity can be tentatively proposed. Firstly, in order to protect a nutrient supply from a dead insect, the bacteria might produce anti-nematocides to prevent saprophytic nematodes gaining access. Alternatively, to become a symbiont, the bacterial strains may have once been pathogens of these nematodes and evolved towards a less hostile symbiotic relationship. The nematocidal activity may be an evolutionary throwback from the original pathogenic relationship, in which case it may be expected to be widely present amongst bacteria which have evolved in this way.

A first aspect of the present invention is the use of bacterial strains to control a target nematode, characterised in that in nature the bacterial strain is associated symbiotically with an entomopathogenic nematode.

As discussed in more detail below, the bacterial strains may be used in the methods of the present invention *per se*, or they may be used as a source of nematode control agent. The nematode control agent can be derived directly, or be prepared and utilised through recombinant DNA techniques, optionally via a host cell.

The target nematode will generally be different to the nematode with which the bacterial strain is found symbiotically in nature.

By means of the present invention employing bacteria or a nematode control agent, it becomes possible to control nematodes, in the sense of, to prevent or retard the effect that the nematode has on other organisms such as animals or more preferably plants, or to reduce the number of nematodes or nematode eggs in an area of interest, or to alleviate or cure a disease caused by nematodes. Control may be at the level of larval nematodes or nematode eggs, or may inhibit the motion, feeding or infectivity of adult nematodes. Nematocidal control may be employed to kill the nematode target. Such controlling activity can be assessed as shown in the Examples below.

PREFERRED EMBODIMENTS

The present invention provides a composition for the control of parasitic nematodes which comprises as an effective agent a species of bacteria which is a symbiont of an entomopathogenic nematode, or engineered bacteria having such activity, or a nematode control agent derived from natural or engineered bacteria.

Correspondingly, the present invention also provides a method of nematode control employing such a composition.

The bacterial species is typically of the genera *Xenorhabdus* or *Photorhabdus*, preferably the genus *Xenorhabdus*, for instance the species *Xenorhabdus bovienii*. Examples of particularly preferred bacteria include:

Xenorhabdus bovienii strain H31 deposited with NCIMB under accession number NCIMB 40985 on 20 January 1999;

Xenorhabdus bovienii strain I73 deposited with NCIMB under accession number NCIMB 40986 on 05 November 1998; and

Xenorhabdus strain C42 deposited with NCIMB under accession number NCIMB 41004 on 05 November 1998.

The nematode control agent can be a peptide derived from a symbiont of an entomopathogenic nematode or an engineered bacterium has functional activity against a nematode. The peptide nematode control agent can be produced from a nucleic acid derived from a symbiont of an entomopath nematode or an engineered bacterium and which encodes such a peptide. The peptide can be an oligopeptide or a polypeptide, notably a protein. In one version, the nematode control agent is a toxin with toxic activity against nematodes, but the nematode control agent can have other activity.

The nucleic acids of this invention can be employed in a method of producing a peptide comprising the step of causing or allowing the expression from a nucleic acid of this invention in a suitable host cell.

The nucleic acid can comprise a natural nucleotide sequence or a degeneratively equivalent sequence, and functional variants thereof. Variants include homologous variants encoding a peptide which is a nematode control agent, the nucleic acid having 70% or more DNA sequence identity and/or the peptide having 70% or more amino acid sequence identity. Especially preferred nucleic acids in p 13-1f and p 14-2f and variants thereof.

The present invention extends to nucleic acids having a sequence which is a derivative by way of addition, insertion, deletion or substitution of one or more nucleotides. The nucleic acid can contain longer expressed sequences such that the nematode control agent is expressed as a fusion protein.

Nucleic acids complementary to the nucl ic acid ncoding a nematode

control agent are also part of this invention.

Nucleic acids for use as a probe or primer having a nucleotide sequence of at least 15, 18, 21, 24 or 30 nucleotides, which sequence is present in, or complementary to, the nucleic acid encoding nematode control agent are further provided by this invention. In this respect, the invention extends to a method for identifying or cloning a nucleic acid for nematode control agent which method employs such a nucleic acid probe.

A method provided by this invention comprises the steps of:

(a) providing a preparation of nucleic acid from a bacterium,

(b) providing a probe,

(c) contacting nucleic acid in said preparation with said probe under conditions for hybridisation of probe to any said gene or homologue in said preparation, and,

(d) identifying said gene or homologue if present by its hybridisation with said probe.

The hybridisation conditions can be selected to allow the identification of sequences having 70% or more sequence identity with the probe.

In one embodiment, the method comprises use of two primers to amplify a nucleic acid encoding a nematode control agent, at least one of the primers having a conserved nucleotide sequence of at least 15 nucleotides.

A method is further made possible by this invention comprising the steps of:

(a) providing a preparation of nucleic acid from a bacterium,

(b) providing a pair of nucleic acid molecule primers, at least one of which is a primer,

(c) contacting nucleic acid in said preparation with said primers under

conditions for performance of PCR,

(d) performing PCR and determining the presence of absence of an amplified PCR product.

Additionally, the invention provides a recombinant vector comprising a nucleic acid of this invention. The vector is preferably capable of replicating in a suitable host such as *E. coli* or in *Xenorhabdus*. The vector can be a baculovirus. In a preferred feature, the nucleic acid is operably linked to a promoter or other regulatory element for transcription in a host cell. Vectors can further comprise any one or more of the following: a terminator sequence; a polyadenylation sequence; an enhancer sequence; a marker gene; a sequence encoding pesticidal material derived from *Bacillus thuringiensis*.

The vector can be a plant vector.

The vector of this invention can be introduced into a cell. Thus, a method for transforming a plant cell comprises the step of causing or allowing recombination between the vector and the plant cell genome to introduce the nucleic acid into the genome. The nucleic acid can be incorporated into chloroplast DNA, or into mitochondrial DNA.

Host cells comprising a vector are also part of this invention. The host cell can be a plant cell, which may be in a plant.

To this end, a method for producing a transgenic plant comprises the step of regenerating a plant from the transformed cell. In turn, plants of this invention extend to the progeny of such plants.

Examples of plants of this invention include crop speci s which can be

protected, notably maize, cotton, soya, rice, *Brassica* species, tomato, potato, sugar beet, barley, soybean, peanut, onion, rye, wheat, corn, banana, raspberry, bean. Decorative and other plants are also possible, e.g. rose.

A part of the propagule of the plants is also envisaged by this invention.

A method of influencing or affecting the toxicity of a cell such as a plant cell is provided where the method includes causing or allowing expression of a heterologous nucleic acid of this invention within the cells.

In a further aspect, the invention involves the use of a material selected from: an *X. bovienii* strain, a nematode control agent; a nucleic acid; a host cell; a plant; a peptide; or a composition of the invention, for the control of a pest, especially where the pest is a nematode and the material is used to control the nematode.

The present invention extends to control of helminthiasis in humans and other animals including domesticated animals such as swine, sheep, horses, cattle, goats, dogs, cats and poultry. The nematodes to be controlled include *Haemonchus, Trichostrongylus, Ostertagia, Nematodirus, Cooperia, Ascaris, Bunostomum, Oesophagosromuni, Chaberria, Trichuris, Strongylus, Trichonema, Dictyocaulus, Capillaria, Heterkis, Toxocara, Ascaridia, Oxyuris, Ancylostoma, Uncinaria, Toxascaris, Caenorhabditis* and *Parascaris*.

Target nematodes may be selected from the genera *Aphelenochoides, Anguina, Bursaphalenchus, Criconemella, Melodigyne, Ditylenchus, Globodera, Heliocotylenchus, Heterodera, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, Trichodorus, Xiphenema,* and *Caenorhabditis*.

The compositions of this invention can be used in conjunction with *Bacillus*

thuringiensis or pesticidal materials derived therefrom.

In a further aspect, there is provided an antibody or fragment thereof, or a polypeptide comprising the antigen-binding domain of the antibody, capable of specifically binding a peptide of this invention.

Such an antibody or fragment can be obtained by immunising a mammal with the peptide, and is useful in a method of identifying and/or isolating a nematode control agent comprising the step of screening candidate polypeptides with a polypeptide comprising the antigen-binding domain of the antibody of claim.

Some further aspects of preferred embodiments of the invention will now be discussed.

Bacterial strains

These can be derived from any entomopathogenic nematode. Preferred species are *Xenorhabdus* and *Photorhabdus*.

Potential sources of bacteria for use in the methods of the present invention may be identified by any preferred method. For instance, entomopathogenic nematodes can be isolated using an insect baiting technique such as that described by Bedding & Akhurst (1975) Nematologia 21: 215-227. Bacteria from nematodes identified as being pathogenic to the insect are isolated, cultured, and used as a source of nematocidal agent, e.g. by analogy with the methods used in the Examples below. Preferably *Xenorhabdus* or *Photorhabdus* species are used.

The preferred bacterial strains include ones which have the characteristics of

strain C42, I73 or H31 isolated by the present inventors. This *Xenorhabdus* strain has the following characteristics: rod shaped; motile; non-bio luminescent; blue on NBTA; produces antibiotics; resistant to ampicillin; forms circular colonies; has convex morphology; white colour.

This strain was presumptively identified as belonging to the genera *Xenorhabdus* since it was isolated from an insect killed by an entomopathogenic nematode and had the above characteristics. The strain has been deposited at the NCIMB (23 St Machar Drive, Aberdeen, AB24 3RY, Scotland) by the applicants under accession number NCIMB 41004 on 20 January 1999.

Further preferred strains of the present invention are two strains of *X. bovienii* designated H31 and I73 which have also been deposited under the terms of the Budapest Treaty at the NCIMB under the accession numbers NCIMB 40985 and 40986 respectively. These share characteristics of C42 in that they are rod-shaped; motile; non-bioluminescent; blue on NBTA; produce antibiotics; resistant to ampicillin; form circular colonies; and have convex morphology. The strains were identified as belonging to the species *X. bovienii* when compared to the *X. bovienii* type strain T228 using Restriction Analysis of the complete 16S rRNA gene and partial sequence analysis.

Target nematodes and diseases

The group of diseases described generally as helminthiasis is due to infection of an human or other animal host with parasitic worms known as helminths. Helminthiasis is a prevalent and serious economic problem in domesticated animals such as swine, sheep, horses, cattle, goats, dogs, cats and poultry. Among the helminths, the gr up of worms described as nematodes causes

widespread and often at times serious infection in various species of animals. The most common genera of nematodes infecting the animals referred to above are *Haemonchus*, *Trichostrongylus*, *Ostertagia*, *Nematodirus*, *Cooperia*, *Ascaris*, *Bunostomum*, *Oesophagosromuni*, *Chaberria*, *Trichuris*, *Strongylus*, *Trichonema*, *Dictyocaulus*, *Capillaria*, *Heterakis*, *Toxocara*, *Ascaridia*, *Oxyuris*, *Ancylostoma*, *Uncinaria*, *Toxascaris*, *Caenorhabditis* and *Parascaris*. Certain of these, such as *Nematodirus*, *Cooperia*, and *Oesophagostomum*, attack primarily the intestinal tract, while others, such as *Dictyocaulus* are found in the lungs. Still other parasites may be located in other tissues and organs of the body.

The bacteria and encoded toxins of the invention may be used as nematocides for the control of the nematodes and diseases discussed above. More preferably, however, they are used to control soil and plant parasitic nematodes. Particular crop species which can be protected include tomatoes, potatoes, sugar beet, barley, soybean, peanut, onion, rye, wheat, corn, banana, raspberry, beans. Decorative and other plants may also be treated e.g. rose.

Target nematodes may be selected from the genera Aphelenochoides, *Anguina*, *Bursaphalenchus*, *Criconemella*, *Melodoigyne*, *Ditylenchus*, *Globodera*, *Helicotylenchus*, *Heterodera*, *Pratylenchus*, *Radopholus*, *Rotelynchus*, *Tylenchus*, *Trichodorus*, *Xiphenema*. A further organism used in certain of the Examples below is *Caenorhabditis elegans*. Other target organisms and plants are discussed by Agrios in "Plant Pathology - 3rd Ed" Pub Academic Press Inc, see Chapter 15 therein.

As stated above, the target nematode will generally be different to that with which the bacterial strain is found in nature.

Methods of use of bacteria

The bacteria may be used in any appropriate method which brings them into contact with the target nematode, preferably such that they, or their products, are ingested or absorbed by the target nematode.

In particular, regarding plants, the bacteria may be formulated in a variety of ways so as to enhance stability. For instance they may be employed in admixture with substrates to protect the cells.

The mixture can be spread over, ploughed into or otherwise mixed with nematode infected or potentially infected soil.

Regarding animals, bacteria intended for enteric inoculation can be mixed with carrier material that is suitable for ingestion by the intended animals.

Isolation of agent

Nematode control agents of the present invention, which may be proteinaceous, or nucleic acids encoding them, may be isolated and/or purified from the C42, I73 or H31 bacteria described above, in substantially pure or homogeneous form, or free or substantially free of other materials from the bacterial strain of origin. Where used herein, the term "isolated" encompasses all of these possibilities.

Methods of purifying proteins from heterogenous mixtures are well known in the art, e.g. selective precipitation, proteolysis, ultrafiltration with known molecular weight cut-off filters, ion-exchange chromatography, gel filtration, etc. A particularly useful initial technique in this regard is ultracentrifugation. Further methods which are known to be suitable for

protein purification are disclosed in "Methods in Enzymology Vol 182 - Guide to Protein Purification" Ed. M P Deutscher, Pub. Academic Press Inc. Other references which outline techniques commonly used by those of ordinary skill in the art include "Protein Purification - principles and practice" Pub. Springer-Verlag, New York Inc (1982), and by Harris & Angal (1989) "Protein purification methods - a practical approach " Pub. O.U.P. UK.

Nematocidal activity may be assessed using a spread assay as discussed below.

The C42, I73 or H31 agent may be wholly or partially synthetic. In particular they may be recombinantly produced from nucleic acid sequences which are not found together in nature (do not run contiguously) but which have been ligated or otherwise combined artificially.

For instance, in the Examples below, nucleic acid encoding toxin(s) from I73 has been expressed in hosts cells using a vector system. Amino acid sequences of 38 different putative I73 toxin(s) are set out in sequence Annex I. These sequences are based on the nucleic acid sequence set out in Fig 2 ('chrim5'), a cosmid clone derived from I73 genomic DNA which conferred nematocidal activity upon *E. coli* cells into which it was introduced (i.e. significantly reduced nematode larval growth and development, and feeding). As detailed below, the entire amino acid sequence as set out in each case may not be required for nematocidal activity. In particular the portion up to the first Met in each sequence may be omitted, as may other portions which may not contribute to the nematocidal activity. Thus, not all the proteins or genes may be required for nematocidal activity, and usually there will be one or more principal proteins, though others may play supporting roles such as in enhancing the activity or encoding other nematocidal activities.

Thus isolated nematocidal agents comprising a polypeptide containing all, or a nematocidal fragment, of any of the depicted I73 sequences, form one aspect of the present invention. Preferred agents include those encoded by p14-2f and p13-1f. Other active variants of these sequences are also encompassed as described below.

Candidate agents for use in this invention to control nematodes extend to those from the bacteria described in PCT/WO 99/22598, as well as the insecticidal toxins and bacteria of PCT/WO 99/42589, PCT/WO 98/08388 and PCT/WO 97/17432, the disclosures of which are incorporated by reference.

Nucleic acids and variants

In one aspect of the present invention there is provided a nucleic acid molecule encoding a nematode control agent of the present invention, for example a toxin, as described above.

The nucleic acid may be derived from the sequence shown in Fig 2 or the complement (or degenerate equivalent) thereof. This sequence (cHRIM5) was itself derived from I73 and identified by its unexpected nematocidal activity. Regions of this sequence believed to correspond to genes of the present invention are described in Fig 3. Isolated nucleic acids comprising one or more of these regions which encode a nematocidal activity are particularly preferred.

In the light of the present disclosure, further nucleic acids of the present invention may be isolated using PCR or southern blotting or other techniques well known to those skill d in the art. This requires the use of two primers to specifically amplify target nucleic acid, so preferably two

nucleic acid molecules with sequences characteristic of the C42, H31 or most preferably an I73 toxin isolated as above are employed. Using RACE PCR, only one such primer may be needed (see "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990)).

Thus a method involving use of PCR in obtaining nucleic acid according to the present invention may include:
(a) providing a preparation of bacterial nucleic acid,
(b) providing a pair of nucleic acid molecule primers suitable for PCR, at least one of said primers being a primer based on a toxin from C42, H31 or I73,
(c) contacting nucleic acid in said preparation with said primers under conditions for performance of PCR,
(d) performing PCR and determining the presence or absence of an amplified PCR product. The presence of an amplified PCR product may indicate identification of a variant.

In a further aspect of the present invention there are disclosed nucleic acids which are variants of the C42, I73 or H31 toxin. A variant nucleic acid molecule shares homology (or identity) with all or part of the C42, H31, or most preferably I73 sequence discussed above.

Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98). Parameters are set, using the default matrix blosum62, as follows:
Gapopen (penalty for the first residue in a gap): -12 for proteins / -16 for DNA
Gapext (penalty for additional residues in a gap): -2 for proteins / -4 for DNA
KTUP word length: 2 for proteins / 6 for DNA.

Homology (similarity or identity) may be at the nucleotide sequence and/or encoded amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares at least about 70%, 75% , 80%, or 85% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology.

Another method for assessing homology at the nucleic acid level is by hybridization screening. One common formula for calculating the stringency conditions required to achieve hybridisation between nucleic acid molecules of a specified sequence homology is shown in Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press:

Tm = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the Tm is 57°C. The Tm of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

Variants of the present invention can be artificial nucleic acids. Alternatively they may be novel, naturally occurring, nucleic acids, isolatable using the information disclosed herein. Thus a variant may be a distinctive part or fragment (however produced) corresponding to a portion of the C42, I73 or H31 toxin. The fragments may encode particular functional parts of the agent or they may be used for probing for, or amplifying, sequences corresponding to C42, I73 or H31 toxin. Sequence variants which occur naturally may include homologs of the C42, I73 or H31 toxin from other

bacteria, including nematode-symbionts. Artificial variants (derivatives) may be prepared by those skilled in the art, for instance by site directed or random mutagenesis (i.e. nucleotide addition, deletion or substitution, optionally to lead to amino acid addition, deletion or substitution) or by direct synthesis. Preferably the variant nucleic acid is generated either directly or indirectly from an original nucleic acid encoding the C42, I73 or H31 toxin.

Changes may be desirable for a number of reasons, including introducing or removing the following features. Sites which are required for pre- or post-translation modification. Changes for codon usage preferences to enhance gene expression in different organisms. Leader or other targeting sequences (e.g. membrane or golgi locating sequences) may be added to the expressed protein to determine its location following expression. All of these may assist in efficiently cloning and expressing an active polypeptide in recombinant form. Other desirable mutation may be random or site directed mutagenesis in order to alter the activity (e.g. host specificity) or stability of the encoded polypeptide. Changes may be by way of conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Also included are active (nematocidal) variants having non-conservative substitutions.

Variant nucleic acids encompass all of these possibilities. When used in the context of polypeptides or proteins they indicate the encoded expression product of the variant nucleic acid i.e. variants of C42, I73 or H31 toxin e.g. variants of the I73 toxin sequences disclosed hereinafter.

Vectors and production of host cells

In one aspect of the present invention, the nucleic acid encoding the nematode control agent is provided in the form of a recombinant and preferably replicable vector.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Sambrook et al (1989) *supra*.

The permitted vectors include, *inter alia*, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally, e.g. an autonomous replicating plasmid with an origin of replication. Illustratively integration can occur into chloroplast DNA or into mitochondrial DNA.

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate optionally inducible promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, yeast, filamentous fungal or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell. The vectors and host cells into which they are introduced may be used to clone or otherwise

identify nucleic acids according to the invention.

The agent may be used as part of a viral vector which is itself pathogenic to nematodes.

Also of interest in the present context are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148). Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809). Suitable promoters which operate in plants include the Cauliflower Mosaic Virus 35S (CaMV 35S). Other examples are disclosed at page 120 of Lindsey & Jones (1989) "Plant Biotechnology in Agriculture" Pub. OU Press, Milton Keynes, UK.

Host cells

The toxin genes or gene fragments encoding the nematocidal agents of the subject invention may be introduced into a host cell, microbial, animal or plant. Expression of the toxin gene in the host cell results, directly or indirectly, in the intracellular production and maintenance of the nematocide.

Thus the present invention also provides methods comprising introduction of such a construct into a plant cell or a microbial cell and/or induction of expression of a construct within a cell, by application of a suitable stimulus e.g. an effective exogenous inducer.

Hosts may be used to assay the activity of particular sequences or

fragments. Hosts can also be used to generate quantities of toxin which can be employed in situ in suitable treated cells, or alternatively with suitable hosts, e.g., *Pseudomonas* viable microbes can be applied to the sites of nematodes where they will proliferate and where they or their products can be ingested by the nematodes. Higher organisms, preferably plants, can also be engineered with the toxin. The result in each case is a control of the nematodes. A host may be selected that can tolerate harsh environmental conditions and then grow when they improve, as illustrated by *Bacillus* species where the spores can exist under environmental extremes.

Characteristics of interest for use as a nematocide microcapsule i.e. a vehicle for the active agent include protective qualities for the nematocide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to nematodes for ingestion; ease of killing and fixing without damage to the toxin; and the like.

Treated host cells

Where the cell is treated, the cell will usually be intact and be substantially proliferative form when treated, rather than in a spore form, although in some instances spores may be employed. Treatment of the microbial cell, e.g. a microbe containing the bacterial toxin gene or gene fragment, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability in protecting the toxin.

Viable hosts

Where the toxin gene or gene fragment is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is preferable that microorganism hosts are selected which are known to occupy the phytosphere (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the nematocide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylophilius*, *Agrobacterium* , *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leuconosroc*, and *Alcaligenes*; fungi, particularly yeast, e.g., genera *Saccharomyces*, *Cryprococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodororula*, and *Aureobasidium*.

Plants as hosts

Nucleic acid encoding the nematocides of the present invention can be introduced into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882,

EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) Plant Tissue and Cell Culture, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. Plant Cell Physiol. 29: 1353 (1984)), or the vortexing method (e.g. Kindle, PNAS U.S.A. 87: 1228 (1990d). Physical methods for the transformation of plant cells are reviewed in Oard, 1991, Biotech. Adv. 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. It has also been used with filamentous fungi (see de Groot et al, 1998, Nature Biotechnology 16: 839-842).

Recently, there has also been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (see e.g. Hiei et al. (1994) The Plant Journal 6, 271-282)). Microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* alone is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g. bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

Generally speaking, following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications, Academic Press, 1984, and Weissbach and

Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989.

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) Current Opinion in Biotechnology 5, 158-162.; Vasil, et al. (1992) Bio/Technology 10, 667-674; Vain et al., 1995, Biotechnology Advances 13 (4): 653-671; Vasil, 1996, Nature Biotechnology 14 page 702).

Combination nematocides

In further embodiments of the invention, bacteria associated with entomopathogenic nematodes or the toxins or products discussed above are used in conjunction with other nematocidal bacteria such as *B. thuringiensis* strains (e.g. from WO 92/19739) or pesticidal materials derived therefrom.

Materials for use in the present invention

The present invention also embraces materials for use in the methods above. These materials include the novel bacterial strains which are associated symbiotically with an entomopathogenic nematode and which are capable of controlling a target nematode. In particular the invention encompasses strain C42, I73 or H31 in isolated or substantially isolated form, or strains having the characteristics of C42, I73 or H31 (including nematocidal activity assessed as below).

Also embraced are compositions and formulations of these bacteria. These may comprise or consist of wettable powders, granules or dusts, mixed with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, methylcellulose, xanthan gum and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells,

peat moss, vermiculite, soil, seeds, other plant tissue and the like). The formulations may include spreader-sticker adjutants, stabilizing agents or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

Bacteria may be mixed with other material while in freeze-dried form, encapsulated in biodegradable or water-soluble material, or otherwise treated to prolong their viability or decrease their levels of metabolic activity during handling. If desired, the carrier material may contain assimilatable nutrient sources to support proliferation of the bacteria.

Also included are purified or substantially purified nematocidal agents (particularly proteinaceous agents) isolated or isolatable from the strains or host cells discussed above.

Thus the invention further discloses nematocidal compositions comprising one or more agents as described above. Such compositions preferably further comprise other nematocidal materials from other *Xenorhabdus* species or non-*Xenorhabdus* species. These other materials may be chosen such as to have complementary properties to the agents described above, or act synergistically with it.

Toxins of the invention for use with animals can be adapted to be administered orally in a unit dosage form such as a capsule, bolus or tablet, or as a liquid drench when used as an anthelmintic in mammals, and in the soil to control plant nematodes. The drench is normally a solution, suspension or dispersion of the active ingredient, usually in water, together with a suspending agent such as bentonite and a wetting agent or like

excipient. Generally, the drenches also contain an antifoaming agent. Drench formulations generally contain from about 0.001 to 0.5% by weight of the active compound. Preferred drench formulations may contain from 0.01 to 0.1% by weight, the capsules and boluses comprise the active ingredient admixed with a carrier vehicle such as starch, talc, magnesium stearate, or dicalcium phosphate. Where it is desired to administer the toxin compounds in a dry, solid unit dosage form, capsules, boluses or tablets containing the desired amount of active compound usually are employed. These dosage forms are prepared by intimately and uniformly mixing the active ingredient with suitable finely divided diluents, fillers, disintegrating agents and/or binders such as starch, lactose, talc, magnesium stearate, vegetable gums and the like. Such unit dosage formulations may be varied widely with respect to their total weight and content of the antiparasitic agent, depending upon the factors such as the type of host animal to be treated, the severity and type of infection and the weight of the host.

When the active compound is to be administered via an animal feedstuff, it is intimately dispersed in the feed or used as a top dressing or in the form of pellets which may then be added to the finished feed or, optionally, fed separately. Preferably, a carrier for feed administration is one that is, or may be, an ingredient of the animal ration. Suitable compositions include feed premixes or supplements in which the active ingredient is present in relatively large amounts and which are suitable for direct feeding to the animal or for addition to the feed either directly or after an intermediate dilution or blending step. Typical carriers or diluents suitable for such compositions include, for example, distillers' dried grains, corn meal, citrus meal, fermentation residues, ground oyster shells, wheat shorts, molasses solubles, corn cob meal, edible bean mill feed, soya grits, crushed limestone and the like.

Alternatively, the antiparasitic compounds may be administered to animals parenterally, for example, by intraluminal, intramuscular, intratracheal, or subcutaneous injection, in which event the active ingredient is dissolved or dispersed in a liquid carrier vehicle. For parenteral administration, the active material is suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety, such as peanut oil, cotton seed oil and the like. Other parenteral vehicles, such as organic preparations using solketal, glycerol, formal and aqueous parenteral formulations, are also used. The active compound or compounds are dissolved or suspended in the parenteral formulation for administration; such formulations generally contain from 0.005 to 5% by weight of the active compound.

Further aspects of the invention include nucleic acids, vectors and host cells containing a heterologous construct according to the present invention, especially a plant or a microbial cell.

Such microbial cells may be treated as described in the methods above. Examples of chemical reagents are halogenating agents. Other suitable techniques include treatment with aldehydes, such as formaldehyde and glutaraldehyde; anti-invectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Bouin's fixative and Helly's fixative (See: Humason, Gretchen L., Animal Tissue Techniques, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host animal. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the nematode control agent.

In all of the compositions discussed above, the nematocide concentration may vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The nematocide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the nematocide while the liquid formulations will generally be from about 16% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^{10} cells/mg, more preferably 10^7 to about 10^9 cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare. The formulations can be applied to the environment of the nematodes, e.g., plants, soil or water, by spraying, dusting, sprinkling, or the like.

In addition to the above the invention includes plant cells which have been transformed with the genes of the present invention, and plants which include such plant cells.

EXAMPLES OF THE INVENTION

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

FIGURES

Fig 1 shows the cHRIM5 cosmid vector and subclones used for sequencing, as described in Example 6.

Fig 2 shows the sequence of cHRIM5 (1-37544 bps).

Fig 3 shows the position and orientation of ORFs in the cHRIM5 sequence.

Fig 4 shows deletions of cHRIM5 tested for nematocidal activity.

Fig 5 illustrates cloning of nematocidal activity in PLEX.

Example 1 - Source of strains C42, I73 and H31

Strain C42 was obtained using an insect entrapment method. Insects which were killed on the surface of a soil sample were observed under a microscope at high magnification. Any that contained high numbers of bacteria and not fungal hyphae were presumed to have been killed by insect parasitic nematodes. The identified presence of nematodes also aids this identification step, but it is not essential. These samples were plated on to NBTA media (see Poinar & Thomas, 1984 Nematodes p238-280 in "Laboratory guide to insect pathogens and parasites" Eds. Poiner & Thomas, Pub. Plenum Press, New York). Any colonies that developed that had characteristic features (e.g. morphology, size, colour) of *Xenorhabdus* or *Photorhabdus* strains were selected. Non-luminescent colonies were presumptively identified as *Xenorhabdus*. The identity of those having nematocidal activity as assessed in Example 3, is further confirmed using 16s rRNA sequence data (see Brunel et al 1997, Applied and Environmental Microbiology 63,2: 574-580).

I73 and H31 strains were obtained in a similar way to strain C42 but they were identified as belonging to the species *X. bovienii* when compared to the *X. bovienii* type strain T228 using Restriction Analysis of the complete 16S

rRNA gene (see Brunel et al, 1997 Applied and Environmental Microbiology: 574-580), and partial 16s ribosomal RNA sequence analysis.

Example 2 - Cell growth and preservation

Subcultures of the *Xenorhabdus* species C42, I73 and H31 were used to inoculate three 9 cm diameter petri dishes containing L agar (10g tryptone, 5 g Yeast Extract, 5 g NaCl and 15 g agar per lt). Plates were incubated for 48 hrs at 26°C and the resulting growth harvested by scraping off bacterial cells and thoroughly resuspending in 40 mls of 5% w/v lactose. The cells were washed once by centrifugation (5000 x *g* for 10 mins), resuspended in 10 mls of 5% w/v lactose, dispensed into 1 ml aliquots and freeze dried (-60°C for 48 hrs) for medium term storage at 2°C. Other stocks were re-suspended in nutrient broth containing 10% w/v glycerol (Protect) and frozen at -70°C.

Example 3- Activity of cells against *Caenorhabditis elegans*

The bioassays were performed by allowing *C. elegans* to feed on live bacterial cell suspensions spread over the surface of Luria broth agar (Luria broth containing 1.2%w/v agar) in segmented square petri dishes (2.0 x 2.0 cm per test well). A minimum of three test wells, each containing 50-100 nematodes were used for each test. Mortalities were recorded after 3 days at 18°C.

C. elegans was cultured on *Escherichia coli* at 18°C on 9 cm diameter LB agar plates. Once the nematodes had colonised the complete plate they were re-subbed on a fresh plate to maintain stocks and the remainder re-suspended in 40 ml LB. The tube was allowed to stand for 15 min and the nematodes settled to the bottom. The concentrated nematodes were removed using a

sterile pipette and placed in 40 mls of fresh LB. The process was repeated 5 more times to wash the nematodes away from the *E. coli* cells. The nematodes were then diluted so that approximately 50 nematodes were present in 50 µl of LB.

The *Xenorhabdus* cells used were cultured in LB at 30°C/100 rpm for 24 hours and 50 µl spread on to the surface of each test well. The control *E. coli* cells were treated in a similar way but incubated at 37°C for growth. After application the wells were air dried for 30 min, and 50 µl of the nematode suspension placed in each well. Again the wells were air dried for 30 min. Plates were incubated at 18°C with 80% relative humidity for 3 days.

Xenorhabdus spp. C42, H31 and I73 gave 95% mortality, as compared with no significant effect for certain other *Xenorhabdus* bacterial strains and *E. coli*. Thus these results clearly show that cells from *Xenorhabdus* C42, H31 and I73 are an effective nematocide.

Example 4 - Cloning of nematode active gene from I73

Total DNA was isolated from I73 using a Quiagen genomic DNA purification kit (cat no. 10243). To isolate DNA, cells were grown in Luria broth (10g tryptone, 5g yeast and 5g NaCl per lt) at 26°C with shaking at 200 rpm to an optical density of 1.5 A600. Cells were harvested by centrifugation at 4000 x *g* and the DNA isolated using Quiagen 100/G tips, as per manufacturer's instructions. The purified DNA was stored at -20°C in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

To obtain a representative I73 library, total DNA was partially digested with *Sau*3A. Approximately 25 µg of DNA was incubated at 37°C with 0.25 units

of enzyme. At intervals of 5, 15, 30, 45 and 60 minutes, samples were removed and heated at 65°C for 10 minutes. To determine the size of the resulting DNA fragments, the samples were separated on a 0.5% (w/v) agarose gel. The samples containing a dominant DNA fragment size of between 30 and 50 Kb were combined and treated with shrimp alkaline phosphatase (Boehringer) for 20 minutes at 37°C. The DNA was ligated into the *Bam*HI site of the Stratagene cosmid vector Supercos1 (scos) and packaged into the *Escherichia coli* strain XL Blue 1, using a Gigapack II packaging kit (Stratagene) following the manufacturer's instructions.

To identify individual cosmid clones with activity to *C. elegans*, single colonies were grown in individual wells of segmented square petri dishes on Luria agar, containing 50 µg/ml ampicillin at 30°C for 24 hours. To each well, approximately 50, mainly L4 and adult *C. elegans* larvae were added in 50 µl of Luria broth. The dishes were incubated at 18°C and examined after 6 days for nematode development.

A total of 600 clones were examined and one coded cHRIM5 was found, which caused significant reduction in larval numbers, with no live L4 and adult larvae observed compared to on average, greater than 40 in all other clones tested.

Example 5 - Activity of cHRIM5, C42, H31 and I73 against *C. elegans*

Clone cHRIM5 was grown in 50 mls LB containing 50 µg of ampicillin per ml at 30°C/200 rpm for 40 hours. C42, H31 and I73 were grown in 50 mls LB at 26°C for 48 hours/200 rpm. Cultures were centrifuged at 4000 x *g* for 10 minutes, washed once and resuspended in 5 mls of PBS (0.05 mM phosphate buffer, 0.125M NaCl). To determine activity, 300 µl of cells were added in triplicate, to 1.2 ml of PBS containing 25, mainly L4 and adult *C.*

elegans larvae in multi well square dishes. As a control, an equivalent amount of XL 1 Blue *E. coli* cells containing Supercos 1 were used to determine nematode survival. The assays were incubated at 18 °C for 7 days before approximate nematode counts and observations were made.

Activity of cells on *C. elegans*

Cell line	No. and size of larvae/square	Cell turbidity
XL 1 Blue/Supercos 1	>100 (all stages)	Clear
XL 1 Blue/cHRIM5	<20(mainly small, L1,2 &3)	Cloudy
C42	<10	Cloudy
H31	<10	Cloudy
I73	<10	Cloudy

Thus cHRIM5, C42, H31 and I73 all gave a reduction in nematode numbers, and in particular cHRIM5 cells significantly reduced larval growth and development. All four strains caused a reduction in feeding (as indicated by the cloudy cell suspensions).

Example 6 - DNA and protein sequences

Plasmid and cosmid DNA for cloning was prepared using the QIAGEN midi system (tip 100, cat. No 12143). Cells were grown in Luria broth (Merck) at 37°C with shaking at 200 rpm for 18 hours. Cells were harvested by centrifugation at 6,000 x *g* and the DNA isolated as per manufacturers instructions. Restriction digestion (Roche, Life Technologies), dephosphorylation (Roche) and ligation (Life Technologies) were carried out using manufacturer's recommended conditions and as outlined by Sambrook et al. Transformation was accomplished using electroccompetant cells and a

BIO-RAD Gene pulser set at 12.5V cm^{-2}. Two µl of DNA was used to electroporate 80 µl of early log phase *E. coli* DH5 alpha cells washed 3 times in sterile water (centrifugation at 6000 x g for 5 mins) and resuspended in 1/100th the original volume in 10% (v/v) glycerol. Luria agar containing either kanamycin or ampicillin at 50 µg ml^{-1} were used to select clones where appropriate.

DNA sequence analysis of cHRIM5 was completed by sequencing a number of sub clones and primer walking, see figure 1 for the supercos vector, where the numbers are kBp. The sub clones used are as follows:

code	cHRIM5 treatment	vector used or remaining
A-380	*Hind*III digestion and self-ligation	deleted scos
B-387	*Bam*HI digestion and self-ligation	pUC 19-*Bam*H1 digestion
C-381	*Sal*I-*Bam*HI digestion	scos
E-391	*Sal*I-*Bam*HI digestion	pUC 19-*Sal*I *Bam*H1 digestion
F-392	*Sal*I-*Bam*HI digestion	pUC 19-*Sal*I *Bam*H1 digestion

Sub clone A-380 was constructed by digesting cHRIM5 DNA with the restriction enzyme *Hind*III and re-ligating fragments, this clone contains a deletion of the insert and scos cosmid DNA as the vector. Sub clone B-387 is a *Bam*HI digestion of cHRIM5 cloned into the plasmid pUC19 also cut with *Bam*HI and dephosphorylated. Sub clone C-381 was obtained by digesting CHRIM5 DNA with *Bam*HI and re-ligating the fragments, this clone contains the scos cosmid as the vector. Clones E-391 and F-392 were obtained by cutting cHRIM5 DNA with *Sal*I and *Bam*HI and ligating these fragments into the vector pUC19 also cut with these enzymes.

Sequencing was conducted using the artificial transposon AT2 (supplied by Perkin-Elmer-Applied Biosystems, Primer Island Transposition kit, cat No.

403015) using the cosmid cHRIM5 and all sub-clones as target DNA. One µg of cHRIM5 DNA was incubated with the transposon AT2 for 1 hour at 30°C in a final volume of 20 µl. After incubation the reaction was stopped by adding 5 µl of 0.25M EDTA, 1% (w/v) SDS, and heat treatment at 65°C for 30 mins. The DNA was desalted by dialysis against water. One µl of the reaction mix was used to electroporate 80 µl of early log phase *E. coli* DH5 alpha cells. Colonies were selected on LB media containing 50 µg/ml trimethoprim. Once inserted the transposon mutants were used to provide a range of positions of primer sites at random intervals throughout the clones. The two primers PI+ and PI- near the end of the transposon were used to generate sequence data. In addition standard primers for the pUC19 and scos vectors were used to generate sequence data at the ends of each clone. DNA for sequencing was prepared using the QIAGEN ion exchange media (qiawell8, cat. No. 17122). Clones were grown in 1 ml of Luria broth containing trimethoprin (50 µg ml^{-1}) for 18 hours. Cells were centrifuged at 13,000 x *g* for 5 mins and resuspended in 350 µl of buffer P1. After 5 mins 350 µl of buffer P2 was added and the samples incubated for 5 mins at room temperature. To this 350 µl of buffer P3 was added and the samples left on ice for 15 mins. After centrifugation at 13,000 x *g* for 15 mins the samples were loaded on the Qiagen column under vacuum, and washed with buffer QC. DNA was eluted with buffer QF (500 µl) at 50°C and isopropanol precipitated (0.8 vol). After centrifugation at 13,000 x *g* for 30 min, DNA was washed with 70% (v/v) ethanol and air dried for 10 mins. The final pellet was resuspended in 10 µl of water. Cycle sequencing reactions using the Perkin-Elmer Applied Biosystems division Big Dye reaction kit (cat No. 4303149) were prepared using standard conditions for plasmid and cosmid sequencing. Samples were analysed on ABI Automated Sequencers. DNA sequences were assembled using the DNA* software. The complete sequence of cHRIM5 was obtained by primer walking to join the final DNA contigs together. The final sequence of cHRIM5ed2 is shown

in Figure 2. Analysis of the DNA using the software Clone indicated a number of ORF illustrated in Figure 3 and 4. Corresponding protein sequences are also presented at Annex I.

Example 7 - Fragments that encode nematocidal activity

To identify smaller fragments that encoded nematocidal activity, a series of sub-cloning experiments were performed using *E. coli* DH5 alpha. Qiagen midi and miniprep methods, restriction and ligations were used as for previous examples. Nematicidal activity of all constructs was determined as described in Example 4. In Figure 4, we show the deletions of cHRIM5 tested for nematicidal activity. Restriction sites and genes are indicated. Size in base pairs indicated on the map line. A cHRIM5, B cHRIM6, C cHRIM7, D cHRIM8, E cHRIM8, F cHRIM10, G *Nde*I deletion of cHRIM8, H Approximate positions (arrows) of three AT2 transposon insertions (tn58, tn26, tn43) in cHRIM9.

The cosmid cHRIM5 (figure 4A) was digested with the enzyme *Sal*I and religated. The resulting sub clone cHRIM6, illustrated in Figure 4B showed nematicidal activity. cHRIM6 was digested with the enzyme *Sma*I and religated, producing sub-clone cHRIM7 (Figure 4C). CHRIM7 was digested with *Bgl*II and the kanamycin resistance gene block (*nptII*, Pharmacia) cut with *Bam*HI was ligated into it. After selection on LB containing kanamycin (50μg ml^{-1}) and ampicillin (50μg ml^{-1}) the clone was digested with *Sal*I and religated, in effect creating a deletion from the *Sal*I site to the *Bgl*II site of cHRIM6 to generate CHRIM8 (figure 4D). By cutting cHRIM8 with *Nru*I a further deletion was made to create cHRIM10 (figure 4F). All the above clones maintained nematicidal activity.

Deletion of cHRIM8 with *Nde*I, removed a portion of the p14-2f gene (figure

4G), this reduced nematicidal activity. This indicates that the p14-2f gene or protein are important for nematocidal activity. Transposon mutagenesis of cHRIM9 (a clone very similar to cHRIM7 but deleted with *Nar*I rather than *Sma*I) with the artificial transposon AT2 (Perkin Elmer Applied Biosystems) resulted in a number of inserts within this clone (figure 4H). Insert cHRIM9-tn43 was restriction mapped to an approximate position of bp 20,700 (on cHRIM5) within the p20-9r gene, this mutant retained nematicidal activity. This indicates that this gene is not essential for activity. Insert cHRIM9-tn58 mapped to an approximate position of bp 13,400 (on cHRIM5), within the p13-1f gene, nematicidal activity was reduced. This indicates that this gene, region of DNA or the blocking effect of the transposon in this position is important for activity. Insert cHRIM9-tn26 was restriction mapped to approximate position of bp 15,000 (on cHRIM5) within the p14-2f gene, nematocidal activity was reduced. This indicates that this gene, region of DNA or the blocking effect of the transposon in this position is important for activity.

Clone cHRIM6-tn43 was digested with *Bgl*II and *Not*I and cloned into the vector PLEX (Invitrogen cat. No. K450-01) cut with *Bam*HI and *Not*I. The *E. coli* strain used was GI742 supplied by Invitrogen. The resulting plasmid insert (PLEX-*Bgl*II/tn43, Figure 5) places the p14-2f and p13-1f genes under the control of the bacteriophage Lambda P_L promoter. Figure 5 illustrates the cloning of DNA encoding nematocidal activity in the expression vector PLEX, where: A, plasmid clone; B, insert and gene locations; Tpr, trimethoprim resistance; Apr, ampicillin resistance; P_L, bacteriophage lambda PL promoter; *, plasmid joins to form a circular molecule; **, incomplete genes. Selection of colonies on RMG media (described in the Invitrogen manual) containing ampicillin (50 μg ml^{-1}) and trimethoprim (50 μg ml^{-1}) prevents expression from the P_L promoter. Colonies were then cultured on LB containing Trimethoprim (50μg ml^{-1}) in 2.0 cm^2 wells for

nematocidal tests. The clone was active. This indicates that genes within this fragment have nematocidal activity. The clone PLEX-*Bgl*II/tn43 was digested with *Cla*I and religated, this resulted in a deletion of part of the p13-1f gene, this clone had reduced nematocidal activity indicating the importance of this gene.

All these results indicate that the genes and gene products of p13-1f and p14-2f are important for nematocidal activity. Other smaller genes within the *Bgl*II to *Nru*I sites of cHRIM10 and PLEX-*Bgl*II/tn43 may also be essential. In addition genes outside this region within the remaining cosmid clone (cHRIM5) may also encode products with nematocidal activity, or may enhance the nematocidal activity of genes in the smaller region (*Bgl*II-*Nru*I of cHRIM10 and PLEX-*Bgl*II/tn43).

Example 8 - Field trials

Activity of strains selected in accordance with the above methods, or from depositary institutions which include bacteria which in nature are associated symbiotically with entomopathogenic nematodes, may be further assessed in field trials as follows.

Symbiotic bacteria in the absence of their nematode host can be inoculated into one or more portions of a field which is infested with nematodes, or into containers containing unsterilised soil from such a field. The bacteria can be inoculated onto the roots of plants, or into seeds. Periodically treated and untreated areas or containers can be assayed for nematode larva, egg, or cyst counts and for the presence of the inoculated bacteria by methods well known to those skilled in the art. A reduction in the number of nematode counts in areas in which the symbiote bacteria are present indicates control of the nematodes otherwise found in th untreated areas or samples.

Annex I - amino acid sequences

SEQ ID NO:1

P0-0f

ISWFATGIPTVDALLAEEFWHGDKQAFPPFTCRFTHFDPDKEQDVTLVPSTEEAYWLHRA
LQGQPLHSEVYGDDGTAQAGIPYTVMDSRPQVRLLTGLPGNSPTVWPSVIEQRTWQYERI
ADDPQCHQQVVLNSDRYGFPRETVDIAYPRRPKPAVSPYPDTLPATLFDSSYDEQQQQLR
LTFQRQHYHHLTDTEHQVLGLPDVMRSDAWGYPAARVPREGFTLEDLLAENSLIAPGTPL
TYLGHQRVAYTGTTGTEEKPTRQALVAYTETAVFDELALQAFNGTLSPEALEKKLIESGY
LSVPRPFNTGAESAVWVARQGYTDYGGSEAFYRPLAQRTTVQIGKNTLHWDTHYCAVVRM
QDAAGLYTDAAYDYRFLTPVQITDANDNQQHITLTALGQVSSGRFWGTEEGTPQGYTPPE
DRPFTPPSSVAEALDLKPDLPVANCMVYAPLSWMPLAHTYQEYIAGFTWQALLDAGVVTE
DKRVCALGFRRWVQRQGIVLNGQALADSREPVHVLTLATDRYDTDPDQQLRKSVTYSDGF
GRLLQSAVYHAPGEAWQRAADGSLITDAKGAPLVAHTATRWAVSGRTEYDGKGQPVRTYP
PFFLNAWQYLSDDSARQDLNADTHRYDPLGREYQVRTAKGYLRQNRLTPWFVVNEDENDT
LS

SEQ ID NO:2

P1-2r

YLPQRGQCDMLLVVIGIGYLNGGQEAVIIGGIRVQTRRILHTDDRTVMGIPMEGVFANLH
RRPLSQRTVKRLRPAVIGISLTGDPDRRFRTGIEWAWNRQITRLD

SEQ ID NO:3

P2-0f

SHLPARYGGRLTTLSRKGFMTVNRGDNLHQKTPEVTVLDNRGLTVRELRYHRHPNTPTTT
DERITRHRFTLSGQLAHSIDPRLFDLQQTDNTVNPNMIYDTALTGEVVRTRSVDAGNDLI
LNDITGRPVLAINATEVTRTWQYENDTLPGRPLSITEQPAGEAGRITERFVWAGNSQAEK
NSNLAGQCVRHYDTAGLNQTDSIALNGIPLSVTRQLLPDGTDADWQGNNEPAWNDRLAPE
NFTTLSTADATGAVLTTTDAAGNLQRVAYDVAGLLTGSWLRLAGGTEQVIVKSLTYSAAG
QKLREEHGNGVVTTYTYEPETQRLVGIKTKRPQGHAQGTKVLQDLRYEYDPVGNVVKVTN
DAEVTRFWRNQKVVPENTYVYDSLYQLVSATGREMANIVQQSTLLPTPSLIDSSTYSNYS
RTYNYDRGDNLTQIRHSAPATGNSYTTDITVSDHSNRAVLDTLTDDPAKVDALFTAGGHQ
IPLQPGQNLVWTPRGELLKVAPVVRDGQISDQESYRYDAASQRIIKTHVQQTANSSQAQS
TLYLPGLERHTTINGTTVKEVLHVITIGEAGRAQVRVLHWENGKPGAISNNQMRYSYDNL
IGSSGLEVDGDGQIISMEEYYPYGGTAVWTARSQTEADYKTVRYSGKERDATGLYYYGYR
YYQPWAGSWLSADPAGTIDGLNLYRMVRNNPATLDDKNGLAPGNRYVFFPFIHEDRIFRL
ASANVYRTEHNKSDIIAVVEDKALDSKLFTNSIEQFFKKPKGKAILKGSPDIKERLLNNI
VHDLSNMQVGDQLYVNAHGHSAKPFFYSDSGYSKIIMEQLQRGANYVAKDLVNKFKLPEN
ATIKISTCHSAEGKGAHITVTSTGTNEKMRYSSIIENKGEFSRSLAGTMENELIKLQPGR

VRGNVYGYLGATTFYGAKNEKVIHLKDGNLTTGVHEGKLSMFTKKNRFSENIFGLKVKRS
LTRTNFTGSGV

SEQ ID NO:4

p-2-9r

PAAEYVRDFTITCSVPPASRSQLPVSRPATSYATRCRLPAASVVVSTAPVASAVLRVVKF
SGASRSFQAGSLFPCQSASVPSGSSWRVTDSGMPLSAILSVWFSPAVS

SEQ ID NO:5

P3-2r

QRALLNDIGHFAPGGTDQLIQAVIDIGVLRHHFLVAPEAGNLRIVRHFHHVPHRVVLIAQ
VLQHLRPLCMSLWAFGFYANKALGLRLVGVGGHHAVAVLFAQFLTRGGIRQGFHDNLLCP
ARKPQPTASQQACYVIRHTLQVTGRIGGGQYRAGGIRRAQGGEVFRCQPVVPGGFIVSLP
VCVRTIRQQLARDGQRYAVKRNTVRLVQSGGVIVTHALSGQVAVLLRLTVPCPDKTLCDT
ACFASRLFCDTERASG

SEQ ID NO:6

P3-6r

SDRRQTGYAYSADHYRISGRSTVCTVRAGLMNYQCWLQHAATQLSESDSPKRDAEILLGY
VTGRSRTYLIAFDETLISSEELHQLDSLLVRRIQGEPVAYIIGEREFWSLPFAVSPATLI
PRPDTECLVEKALELLPDSPARILDLGTGTGAIALALASERNDCYVTGVDINSDAVMLAQ
HNAEKNAGKLAIHNVNFLQSEWFAAVGNQQFDMIVSNPPYIDERDPHLQEGDIRFEPATA
LIAAQNGMADLQAIVGQARHFLSPNGWLLLEHGWKQGTVVRNLFLEKGYQQIATFQDYGG
NERITIGRWNKNETHS

SEQ ID NO:7

P3-7f

ARRAVRRCGYCTGRTESRVPSVTTRCATAMITLSAAAVWRWTVTDKLSVWKNTTRTGALR
CGRRGVRQRLITRLCVTQARSGMQRGCIITATGITSRGRGAG

SEQ ID NO:8

P5-6r

WQNGGSSSTTPRYLAGCYVWYPCSARLSGNAKSLLAPDGEWMKHTLKSKASGNTFTGRLI
PTGRPTVVTIDKSGANTAALTLLNAEGEPQQGIEIRQNKYLNNRIEQDHRHVKRRIRPML
GFKSFRRAQT

SEQ ID NO:9

P5-7r

ALLFLSESRVMSLIRNAFKLLHYPVDIMAQCVRWSLTYALSLRNLEEMMAKRGIFVDHAT
IPRWVLRLVPLLSKAFRKRKKPVGSRWRMDETYIKVKGQWKYLYRSVDTDGQTDCGDYR

SEQ ID NO:10

P6-3f

VHSPSGAVAPGKFFIENFADTFPAPLPLHPFIDACIQQGFQLLPCLIAIAHSGKQAFECV
LLDRLALQGSQCLQALVLPVGDVNGQTAHGFLLIGYTQTHISTYNGLWLFITQGVRYRFV
RQTFVCRSLSFSEDDCTN

SEQ ID NO:11

P6-3r

RTCRERPRLMDYVLTKAAEADLRAIIRHTRKQWGDAQVRRYITALEQGIARLAVGQGSFK
DMSALFPALRMAHCERHYVFCLPRENAPALIVAIFHERMDLLTRLADRLK

SEQ ID NO:12

P6-6r

PQTIICANVGLCITDKEKTMSRLTIDITDRQHQSLKALAALQGKTIKQYALERLFPGMSD
SDQAWQELKALLDTRINEGMEGKGCGKSIGEILDEELAGSDRA

SEQ ID NO:13

P7-1f

NAHFLIVSKTNVVMSNQDPHNKRDSLFSAPIANLGDWSFDERVAEVFPDMVKRSIPGYSN
IISMIGMLASRFVTPGSQIYDLGCSLGAATLSIRRSINADNCRIIAIDNSPAMIERCRRH
IDSFKASTPVEVIEQNILDTDIQNASMVVLNFTLQFLHPDDRQKILKKIYAGLKPGGVLV
LSEKFNFEDQKIGELLFNMHHDFKRANGYSELEVSQKRSMLENVMRTDSVDTHKSRLKEV
GFQHVEVWFQCFNFGSLLAIKGTEQ

SEQ ID NO:14

P7-9f

TMIDFGNFYQLIAKHPLNHWLDSLPAQLSHWQKTSQHGQFSSWVKILENLPEIKPSHLDL
KNGVIAIHEPDLSKGEKARLHNILKILMPWRKGPFSLYDVEIDTEWRSDWKWERVLPHIS
PLEGKTVLDVGCGSGYHMWRMVGEGAQLVVGIDPTQLFLCQFEAIRKLLGNNQRAHLLPL
GIEQLPELQAFDTVFSMGVLYHRRSPLDHLWQLKNQLVSDGELVLESLVIEGDENQCLIP
GERYAQMRNVYFIPSAKMLKVWLEKCGFVDVRIVDHAATTPDEQRRTEWMKTESLVDFLD
PSDHSKTIEGYPAPLRAVLIARKP

SEQ ID NO:15

P8-4r

SLQIDREKVGLDRYPQPIERLRQPCATCDNHCHSRHQVRFFLLKEKYGAALAPISSQSAI
RYQFQRHTMKKGLFAMASIFSGYCGGELFHLLTDPAHESQ

SEQ ID NO:16

P9-8r

SSFRLNDDLLTNSYSEGFLMIKLEICCYSISCALVAQNAGADRIELSASPLEGGLTPSFG
ALQQSLQRLSIPVHPIVRPRGGDFCYNNMDFEAMKNDVARIRDMGFPGIVFGILSENGHI
DRLRMRQLMSLSGNMAVTFHRAFDMCFNPHVALEQLTELGVQRILTSGQQQNAELGLTLL
KELMQASRGPIIMPGAGVRVSNISKFLEAGMTEVHSSAGKIVPSTMKYRKVGVAMSSDDR
DVDEYSHYSVDGELVESMKGVMSLIKR

SEQ ID NO:17

P10-5r

YFGKNRRFVIYVTLMERNFYGLFNGEEMSHFSKISELQDLVADLAGFEQKLKQFEGHLGL
HFEQYSADHISLRCNESKIADRWRKGFLQCGQLISESIINGRPICLFDLNQPIVLLDWKI
DCVELPYPSQKHYVHQGWEHVELVLPVPPEQLICEAKKLLPQPLPDNFRMKESHPKGKNE
RLPNPILAV

SEQ ID NO:18

P10-7f

GNTVNIQVILSEKISNALIEAGAPTDSEAHVRQSAKAQFGDYQANGVMAAAKKVGIPPRQ
LAEKVVSQLDLQGIASKVEIAGPGFINIFLDKAWVAANIETTLKDEKLGITPVEPQTIVI
DYSAPNVAKQMHVGHLRSTIIGDAAARTLEFLGHKVIRANHVGDWGTQFGMLIAYLEKIQ
NENANDMALADLEAFYREAKKHYDEDEEFAIRARNYVVKLQGGDEYCRKMWRKLVDITMS
QNQETYNRLNVTLTEKDVMGESLYNDMLPGIVADLKQRGIAVKSDGATVVYLDEFKNKEG
EPMGVIIQKKDGGYLYTTTDIACAKYRHETLNASRVLYYIDSRQHQHLMQAWAIVRKTGY
IPESMSLEHHMFGMMLGKDGKPFKTRAGGTVRLSDLLDEAIERADTLIREKNPDMPEDEL
KKVVEAVGIGAVKYADLSKSRTTDYVFDWDNMLAFEGNTAPYMQYAYTRVSSIFKRADID
ENSLTLPVMLNEEREQALATRLLQFEETITTVAREGTPHVMCAYLYDLAGLFSGFYEHCP
ILNADSEELRQSRLKLALLTAKTLKQGLDTLGIQTVERM

SEQ ID NO:19

P11-1r

AQVSNMHLLGDIRCGIIDNDGLRFHWGDTELFIFQGSFYICCNPRFIKKNIDKTWACNFN
FAGNSLQIQLADDFFCQLSRRYSHLFSGSHHTIRLIVTKLCFGRLTDVSFTVGWSASFNQ
RIADFF

SEQ ID NO:20

P12-1r

HARVGVLHIRCRVAFKGQHIIPVENIVCSTALGKICIFHRANPYRFHDFFQFVFWHIWVF
LTNEGIRTLNRFIQQIGQSYCAAGTGFEWFTIFAQHHAKHVVFE

SEQ ID NO:21

P12-5r

YHASFQLCRRLLHTFYSLNTQSIKTLLQSFRCQQSQLQAALAQFFAIGIQDRAVLIETRE
QTGQIVQVCTHNMWRTFTGDGSDRFFKLQQAGCQCLLAFFIQHHRQCQAVFIDIRTFKDR

SEQ ID NO:22

P13-1f

FTLREDSMSDWTGVSTFNVILETGLDNCNIYANGLNMIGVIINITPTDDEGNFVDIDDVT
LNDNIKIVDYIDGSDIDGSDGWFYTGNPNEYNTIPNSQSYSLLKSENSQITQIKRYVSCS
NTSRLRTKSFSAKVTTTSGKVISITQNSINSSRVVINAIDATNFTDDELRTTKETRFENQ
SYTSHKSSTNSLYVHTWTIPRSLKLQNWRWEDYNNGWTWAQSCYYKTGADGGSESTRWLA
AGSIFPPGNYDGLWLDNDIALSGMAHKSYNVDTGINQLSFTRIIGKGFSWVYNISGLDRG
HAVIIIDQYGNKYRILFHAGYENSDPYLSSSIVY

SEQ ID NO:23

P14-2f

VYIKFLKLFRRITMSDNNEFFTQANNFTSAVSGGVDPRTGLYNIQITLGHIVGNGNLGPT
LPLTLSYSPLNKTDIGFGIGFNFGLSVYDRKNSLLSLSTGENYKVIETDKTVKLQQKKLD
NLRFEKDLKENCYRIIHKSGDIEVLTGFNNNAFDLKVPKKLLNPAGHAIYIDWNFEATQP
RLNRIYDDLDGHDIPLLNLEYQGLIKTILTLFPGQKEGYRTELRFLNRQLNSIHNFSLGN
ENPLTWSFGYTPIGKNGILGQWITSMTAPGGLKETVNYSNNNQGHHFPQSANLPVLPYVT
LMKQVPGAGQPAIQAEYSYTSHNYVGGGSNGIWNNKLDNLYGLMTEYNYGSTESRRYKDK
EGHDQIVRIERTYNNYHLLTSECKQQNGYIQTTETAYYAIIGHNFDSQPSQFQLPKTKTE
TWRSADNSYRSEITETTFDESGNPLTKVIKDKKTQKIISPSTHWEYYPPAGEVDNCPPEP
YGFTRFVKKIIQTPYDSEFKDDPEKFIQYRYSLIGSQSHVTLKIEERHYSATQLLNSTLF
QYNTDKSELGRLLKQTECTKGENGKTYSVVHKFTYTKQDDTLQQSHSITTHDNFTIHRSQ
VRSRYTGRLFSDTDTKDIVTQMSYDKLGRLLTRTLNSGTPYANTLTYDYELNNLQDDNRP
PFVITTTDVNGNQLRNEFDGAGRHVSQCLKDSDGDGKFYTIHTQQYDEQGRHHTSTYSDY
LTNGRQQTDPDKVHLSMSKSYDNWGQIANTHWSYGVSEKITVDPITLTATKQLQSNSNNV
QTGKEVTTYTPSQQPIQITLFDEAGHLQSCHTLTRDGWDRVRKETDAIGQCTIYQYDNYN
RVIQITLPDGTIVNRKYAPFSTDTLITDIRVNGISLGQQTFDGLSRLTQSQDGGRVWAYT
YSAGNDQCPSTVITPDGQFIHYQYQPELDDAVLQVASNEITQQFSYNPVTGALLKAVAEG
QSLTPIYYPSGRLKMENINDMKKMSYLWTLRGLENGYTDLTGTIQKISRDTHGRVTQIKD

SSIKTTLNYDDLNRHIGSQVTDLATGHMLTTTVEFDGLNREIGRKLCDSSGHTLDIQQSW
LKTQQLANRIVKLNGVLQRTEQYSYDSRNRLNQYKCDGAECPTDKYGHSIVTQNFTYDIY
GNITACHTTFADGTEDHATFKFANPTDPCQLTEVHHTHPDMPDNIRLKYDKAGRVINITD
NHGNTENFTYDTLGRLQNGQGSVYGYDPLNRLVSQKTDTLDCELYYRETMLVNEVRNGEM
IRLLRTGETIIAQQRASKVLLTGTDSQQSVILTSDKQNLSQEAYSAYGKHKSTANDASIL
GYNGERADPVSGVTHLGNGYRSYDPTLMRFHTPDSLSPFGAGGINPYSYCLGDPINRSDP
SGHLSWQAWTGIGMGIAGLLLTIATGGMAIAAAGGIAAAIASTSTTALAFGALSVTSDIT
SIVSGALEDASPKASSILGWVSMGMGAAGLAESAIKGGTKLATHLGAFAEDGENALLKST
SESSRIKWGVTRSLDREIVRNEEGQVIKDHSRGYTDNFMGKGEQAILVHGDKDGFLYHTE
GNKHNGKGPYTRHTPEQLVDYLKDNNIVDLTQGGDKPVHLLSCYGKSSGAADKMAKYINR
PVIAYSNKPTISQGLARIERKDFFLKSTYHSYDPRKIILGRTEKTVKPKTFRP

SEQ ID NO:24

P17-6r

LCYGHICLSGIPHRHIYIGSTYYGNRKSTVLYAAILHSVSLFYLLIAVFSASSAGYLTYG
LSYHTISVQFLGLSHQIPLLLSTYDQSLNLLLDYQYGDSGHRNLE

SEQ ID NO:25

P17-8r

SAQCIVGKVFRISMVISDIYYSTSLIIFQPDIIRHIWMSVVYLCQLAWVSWVGKFEGSMV
FCPICECGVTGGDIAIDIISKILCDYAMAIFVCRAFRTVTFILVQPITGIVRVLFCTLQY
SIQFHYSIC

SEQ ID NO:26

P18-7r

PSSLRTISLSKLLVTPHFILELSEVDLSKAFSPSSANAPRCVASLVPPLMADSANPAAPI
PIETHPSIEDAFGEASSSAPLTIDVISDVTLSAPNASAVVEVEAIAAAIPPAAAIAIPPV
AMVSSNPAIPMPIPVHACQLK

SEQ ID NO:27

P19-5f

AHCHIALFPCWHNPQYCQQHPDHHSNCHHQFKQEYPPSRQRRENITLTQLPIKHTGIEAG
SQTNRKRQTCMFQRANESKVHQLGQNQGRDRNFYWCFDILT

SEQ ID NO:28

P19-8f

PQSTPSSQNSRQLTPAESSQHQKQKSDHIEIMIPSEAPREYREQLHKATPARNRDVAPNP
SVFDILRDYHWKNFSPVKAAKSSLTPHPVHQKAIPLNDQRNTSMKQSLKPEMRQKLY

SEQ ID NO:29

P20-1r

GKNCINDQGNLPDRYTQNCRPHLTDNPPYGTVTERNPRQYQHADLFQMRKLIGQLQNPSG
NNGPTQRQHWRIAIRSHKQCKNDHTDIEQCRSKSRHRKAVPCIKNCASQRSQRNQKDIRK
RNSK

SEQ ID NO:30

P20-9r

NNTMNLLKSLAAVSSMTMFSRVLGFIRDAIIARIFGAGMATDAFFVAFKLPNLLRRIFAE
GAFSQAFVPILAEYKNQQGDEATRTFIAYISGMLTLILAIVSVIGVIAAPWIIYVTAPGF
TDTPDKFVLTRDLLRITFPYIFLISLASLAGAILNTWNRFSVPAFAPTLLNVSMIIFALF
VAPYCNPPVLALGWAVVAGGVLQLAYQLPHLKKIGMLVLPRISFRDSAVWRVIRQMGPAI
LGVSVGQISLIINTIFASFLVSGSVSWMYYADRLMELPSGVLGVALGTILLPSLAKSFSS
GNHEEYRKLMDWGLRLCFLLALPCAVALGILAEPLTVSLFQYGHFSAFDAEMTQRALIAY
CFGLMGLIVVKVLAPGFYSRQDIKTPVKIAIATLILTQLMNLAFVGPLKHAGLALSIGLA
ACFNASMLYWQLRKRDIFTPLAGWGIFLFKLVVAIAVMVGVLLAVLWVMPAWEQGNMAMR
LLRLMGVVIAGAGSYFAVLALMGFRLKDFAHRGLQ

SEQ ID NO:31

P21-7r

AIILIRDKLSRIFSRQISGEGMFGYRSASPKIRFITDRMVVRLVYERDAYRLAEYYSENK
DFLKPWEPTRDGSFYQPSGWTNRLNYIAELQRQNATFNFVLLDSDEREIMGVANFTNVVR
GAFHSCYLGYSLAEKLQGQGLMYEALQPAIRYMQRYQRMHRIMANYMPHNHRSGNLLKKL
GFEQEGYAKNYLMIDGVWQDHVLTALTDDAWGKVGL

SEQ ID NO:32

P21-8f

WCAMSLVSQARSLGKYFLLFDNLLVVLGFFVVFPLISIRFVEQLGWAALIVGFALGLRQL
VQQGLGIFGGAIADRFGAKPMIVTGMLLRALGFALMAMAHEPWILLLSCVLSGLGGTLFD
PPRAALVIKLTRPHERGRFYSILMMQDSAGAVVGALIGSWLLQYDFNIVCWIGASIFVLA
ALFNAWLLPAYRISTIRTPIKEGMMRVIRDRRFLYYVLTLTGYFVLSVQVMLMFPIIIHE
ITGTPTAVKWMYAIETAISLTLLYPIARWSEKHFRLEQRLMAGLFLMSICMFPIGWVNQL
HTLFGLLCLFYLGLVTADPARETLSASLSDPRARGSYMGFSRLGLALGGAIGYTGGGWLY
DTGRDLNMPQLPWILLGLSGLITIYALHRQFNQKKIDPVMLGRH

SEQ ID NO:33

P23-1f

KGANMKRFFLGAALVLVGLVSGCDQFKDFSINEGLMNDYLLKKVHYQKKISIPGIANANI
TLGDLSSQIGRQDPEKIELSTQAKVQLATLLGTIQADMKLTIKAKPVFDAEKGAIFVKGL
EIVDYQTTPEKAAAPVKALIPYLNTSLSEFFDTHPVYVLNPEKSKAEAAASQFAKRLEIK
PGKLVIGLTDK

SEQ ID NO:34

P24-4r

QVALQHGRRLGTITLFDNLLGLNQVMNEFSIVCRILGTLFNRAPQDPVLQPLITMIAEGK
LKQAWPLEQDEWLDRLQQNSELSVMAADYHALFTGESASVAVCRSDYTDGEESEVRQFLT
ERGMPLSDTPADQFGSLLLAVSWLEDQAAEDEIQAQITLFDEYLLPWCGQFLGKVEAHAT
SGFYRTLAIVTREALQALRDELESE

SEQ ID NO:35

P25-3r

DCMNIIFFHPSFNTDEWIQGIQARLPDAKVRQWVSGDQEPADYALVWQPPYEMLANRQGL
KGIFALGAGVDAIFKQESKNPGTLLADVPLIRLEDTGMGRQMQEYAITSVLHYFRRMDEY
KRYQEQRLWNPIAPHNRKEFVIGVLGAGILGRSVIGKLMEFDFNVRCWSRTSKQLDSVES
FYGKEQLGDFLSGCKVLINLLPDTPDTRGILNLSLFSQLKSGSYVINLARGAQLVEQDLL
VAIDKGYIAGATLDVFAEEPLSNMHPFWTHPRINVTPHIAANTIPEAAMDVICENIRRMV
QGEMPTGLVDRVRGY

SEQ ID NO:36

P26-0f

KTSQGFTSTTCSNGNVLKICGLITPCSSLIQRTYPNNMTIGIFSKESTAKNFGMGFLYYF
DLRVLSPFFKAPINIFTGWQHNTNFRKSRNSTIRLCSSTPNSKQYFTTSRKCHITGAGKY
RFSIENCFIKSG

SEQ ID NO:37

P27-0r

YSAGCSTVLKSSLNLQCDTFNCESFVMLTLNFSTSVNAKPSHIWAHYVDFDLRKKWEVDL
EYFQFEGEVKTGQYGRMILSGMPEIRFYLSNIEVNKEFTDQVNLPQMGILTFRHQIITDE
NNMACRVQVTVSFEPDANIPAVQAESFFKQGTQDLVESVLRLKSVVETVSPKPNLQLVYV
SDIESSTAFYKTIFNAEPIFASSRYVAFPAGGEVLFAIWSGGAKPDRAIPRFSEIGIMLP
SGKDVDRCFEEWRKNPEIKIVQEPHTEVFGRTFLAEDPDGHIIRVCPLD

SEQ ID NO:38

P27-8r

KGNQITMILYKGSKNYLFNQLNYDSCVLLEVDESVNLNGWDELSRAQRLLFLMEILRRYH

FPVQGKVLAQKLNISLRTLYRDIASLQAQGAIIEGEPGIGYVLRPGFVLPPLMFTQNEIE
ALALGANWVAKRADPQLKESANNAISKIAAVIPAELKQMLEASSLLIGPAATAVQPVVEI
QQIRQAINTRHKITLAYLDIKDIPSERTIWPFALGYFENISIVIGWCELREEFRHFRSDR
IMRLKIENQCYPRSRQVLLKEWRAMEKISR

SEQ ID NO:39

P27-9f

RKMTIYDLKPRFQNLLRPIVIYLYKQGITANQVTLTALFLSIFAGSLLSLFPSPHLYWLL
PVFLFIRMALNAIDGMLAREHNQKSHLGAIYNELGDVISDVALYLPFCLLPDVNSLSLLI
ILFLTILTEFIGVLAQTIGASRRYDGPIGKSDRAFIFGAYGLIIAIFPLALGWSISLFAF
MIILLLVTCYQRVVKALREIRLAEQSHSK

SEQ ID NO:40

P28-5f

GVNMTPQLDQRIAEEHYFTTSDNASLFYRYWPQQQANPDRAIIIFHRGHEHSGRIQHVVD
GLDLPDVPMFAWDARGHGKTEGPRGYSPSMGTSIRDVDEFVRFIATQYGIAMENIVVIGQ
SVGAVLVSAWVHDYAPKIRAMILAAPAFDIKLYIPFATQGLQLMQKARGIFFVNSYVKAR
YLTHDETRIASYNSDPLITREIAVNILLDLYQTAERVVKDAAAITLPTLLFISGSDYVVN
KKPQHQFYQQLNTPIKEKHVMDGFYHDTLGEKDRHLVFDKIRVFIERIFALPRYQHDYSQ
EDTWSHSADEFRTLSTSLPCLCPKKLSYQLMRKVMSTHWGRTSEGVCIGLKTGFDSGSTL
DYVYRNQPQGKGILGRILDKHYLNSIGWRGIRQRKIHIEMLIRHAIRSLREQNMPVHMVD
IAAGHGRYILDAINDFSKVDSILLRDYSEINVNQGQAYIEERDLTDKIRFIIGDAFNAES
ISSITPAPTLGIVSGLYELFPDNNLLRNSLRGFADVMTENGYLVYTGQPWHPQIEVIARV
LSSHRDSQPWIMRRRTQGEMDALVEAAGFEKLYQLTDNWGIFTVSIAKRVHR

SEQ ID NO:41

P28-5bf

HHNSINVLLKNIISPHQIMLLCFTVTGHNNRPIQTERSLFFTVVMSTQDVSSMSLTDSIC
LMFLCSRGMPVDTVRQKGRAVTAHPWERRFVMLMNLSDLLPLSTASPWKISWLSARVSER
Y

SEQ ID NO:42

P30-3f

INKYKMEHHMHSSLDSRRRLWLTGVIWLLFLAPFFFLTYGQVNQFTAQRSDVGTVMFGWE
HNIPFWSWSIIPYWSIDLFYGISLFICTHRREQWLHGWRLMTASLIACVGFLLFPLKFSF
SRPTTEGLFGWLFNQLELFDLPYNQAPSLHIILLWLLWLRYSAYVSGYWRGLLHIWSVLI
ALSVLTTWQHHFIDVLTGFAVGVILSYLLPVSYRWRWQPNQDRYARKLFGYYLTGSALFA
LIASLLGGSFWILLWPAVSLLMIALGYAGLGSSVFQKQPDGRMSLSARWLLAPYQLGAWL
SYLWFRRKSAPFNHITEGIILGSLPCQPVTAVSVLDITAEWHRRSDARTVNYVCQPQIDL

LPLAPEALQSAVCTLDKLRQQGDVFVHCTLGLSRSAMVVAAWLLKQHPEYDINTVVAILR
KARPHVTFRQTHLDALSQWAKGYL

SEQ ID NO:43

P31-6f

QSCVKPDRMSRSDKHIWMPCLNGQKATYNGEHNMQPENLISKVIIATLKSWRFISTLSAF
SILIATAMLIAVFNTTALNNIALYAVLLFTTLYCQYYCWRTWLDCHYFQILNSSPEKSAE
FDQTLLLIFNKLPQSRTQNDRFNGAIKLLKKATIGLILQWILFFLFLLTLKYSA

SEQ ID NO:44

P32-3f

MNTRKINGIRPFSAFIDSCLKESYSFPRFIRDIIAGITVGVIAIPLAMALAIGSGVAPQY
GLYTAAIAGIVIAMTGGSRYSVSGPTAAFVVILYPVSQQFGLSGLLIATLMSGVILIVMG
LARFGRLIEYIPMSVTLGFTSGIAITIATMQVQNFFGLKLAHIPENYIDKVVALYQALPS
LQLSDTLIGLTTLLVLIFWPKLGVKLPGHLPALIAGTAVMGAMHLLNHDVATIGSSFSYT
LADGTQGQGIPPILPQFVLPWNLPDTHSLDISWNTVSALLPAAFSMAMLGAIESLLCAVI
LDGMTGKKHHSNGELLGQGLGNIAAPFFGGITATAAIARSAANVRAGATSPIAAVVHSLL
VLLTLLVLAPMLSYLPLAAMSAILLIVAWNMSEAHKVVDLIRHAPKDDIIVMLLCLSLTV
LFDMVRRDHYRHCAGITPVYAQNCQYDSNQHVIFNKRGERVIGRTN

SEQ ID NO:45

P33-4r

ESIGAKTSNVNNTSRECTTAAIGEVAPARTLAAERAIAAVAVMPPKKGAAILPNPWPSSS
PLEWCFFPVIPSRITAHSNDSIAPSMAIENAAGSNADTVFQLISRECVSGKFHGRTNWGR
MGGMP

SEQ ID NO:46

P33-5f

LSYSIWSVAITIGIVLASLLFMRKIANMTRISTSSLTSAEKGLLVVRINGPLFFAAAERI
FAELREKSADYQTIIMQWDAVPVLDAGGLHAFQGFVRELGKEKHIVVCDIPFQPLKTLAR
AKVMPIEGELSFYATLPKALKEMAVDYTPEVCASSEKIQGQ

SEQ ID NO:47

P34-3f

CMSDVENDRRTLGSLLHDTEAQHVNHQIVITKVAATVTQDHLVIAAFFEFFNNIAHLPRA
NKLWFFNININHSTGFRHRFNQIGLAGKEGWKLNHIHHIRDWLSLCRLMHVSDNFHAEGLFQ
FLKDFHPLFQPWPTIRADRRTVSLIKRRFKNIRNAQFLCHGDIVLTNPHGQIP

SEQ ID NO:48

P35-0r

LSCIRFIFLLIQQIYLPLTREGISMQQKVVNIGDIKVANDLPFVLFGGMNVLESRDLAMR
ICEHYVTVTQKLGIPYVFKASFDKANRSSIRSYRGPGLEEGMKIFQELKQTFGVKIITDV
HEPAQAQPVADVVDVIQLPAFLARQTDLVEAMAKTGAVINVKKPQFVSPGQMGNIVEKFK
EGGNDQVILCDRGSNFGYDNLVVDMLGFGVMQQATQGAPVIFDVTHALQCRDPLGAASGG
RRAQVAELARAGMAVGIAGLFLEAHPDPENAKCDGPSALPLAKLESFLMQIKAIDDVVKN
FPELDTSK

SEQ ID NO:49

P35-8r

VDGIKMKPIVNYEFNNTPLIDGIILVSKIIRPDFPQTLVSEQLTALVEEARQRLSSITDS
KVKLDSLLTLFYREWKFGGANGVYCLSDTLWLDRLLHSRQGSPVSLGTVFTHIAQALGLS
VQPVIFPIQLILRIDLLDQPTWFINPLNGDTLNEHTLDVWLKGNIGPTVRLKKQDLQEAD
NVSLVRKITDTIKVSLMEEKKMELALKASEVVLTFDPDDPYEIRDRGLIYAQLDCNHIAV
SDLSYFVEHCPEDPISEMIKMQINTIEQRLIVLH

SEQ ID NO:50

P36-7r

SDRRQTGYAYSADHYRISGRSTVCTVRAGLMNYQCWLQHAATQLSESDSPKRDAEILLGY
VTGRSRTYLIAFDETLISSEELHQLDSLLVRRIQGEPVAYIIGEREFWSLPFAVSPATLI
PRPDTECLVEKALELLPDSPARILDLGTGTGAIALALASERNDCYVTGVDINSDAVMLAQ
HNAEKNAGKLAIHNVNFLQSEWFAAVGNQQFDMIVSNPPYIDERDPHLQEGDIRFEPATA
LIAAQNGMADLQAIVGQARHFLSPNGWLLLEHGWKQGTVVRNLFLEKGYQQIATFQDYGG
NERITIGRWNKNETHS

SEQ ID NO:51

P37-5r

VEMREMAQEELKEAKIRNEELEQQLQLLLLPKDPDDERNCFLEVRAGTGGDEAAIFAGDL
FRMYSRYAEARRWRVEIISANEGEHGGYKEVIAKVSGDQVYGHLKFESGGHRVQRVPETE
SQGRIHTSACTVAVMPEIPEAELPDISPGDLKIDTFRSSGAGGQHVNTTDSAIRITHLPT
GIVVECQDERSQHKNKAKAMSVLAARIRAAEMRKRQEVEASERRNLLGSGDRSDRNRTYN
FPQGRVTDHRINLTLYRLDEVIEGKLDMLIQPIIIEYQADQLSALSEQD